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ORIGINAL PAPER

MiniMax, a new diminutive *Glycine max* genotype with a rapid life cycle, embryogenic potential and transformation capabilities

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Abstract We developed *Glycine max* cv MiniMax (PI643148) that has a rapid life cycle, short stature and characteristic simple sequence repeat (SSR) markers that could make it useful for mutant screening, functional genomics, genetic mapping and other studies involving soybeans. We demonstrate that MiniMax is able to make somatic embryos (SEs) that rapidly develop into plantlets. Thus, the rapid cycling habit carries over into aspects of plant regeneration. Chimaeras (having transformed roots with untransformed aerial stocks) have been produced rapidly under non-axenic conditions using *Agrobacterium rhizogenes*-mediated transformation. Part of these experiments involved the engineering an enhanced green fluorescent protein (eGFP) reporter cassette outside the multi-cloning site of a plant expression vector, permitting non-invasive visual screening of the transformed roots. The rapid cycling growth habit of MiniMax, its ability to efficiently generate SEs and ability to be transformed should prove useful for basic aspects of *G. max* molecular and genetic research.

Keywords MiniMax · *Glycine max* · Somatic embryogenesis

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Abbreviations

MG	Maturity group
SCN	Soybean cyst nematode
SSR	Simple sequence repeat
GUS	β-glucuronidase
eGFP	Enhanced green fluorescent protein
FMV sgt	Figwort mosaic virus sub-genomic transcript

Introduction

Plant research models such as *Arabidopsis* have been instrumental for studying their development. Their utility comes from a compact size, rapid cycling nature, small genome (Leutwiler et al. 1984) and transformability (Bechtold et al. 1993). However, it

is not always possible to extrapolate what is learned from those models directly into understanding agricultural plant development. For example, much interest is being generated in improving soybean (*Glycine max*) because of its significant agricultural status. However, research is hampered in *G. max*, due to its large size, long lifecycle, large genome and recalcitrance to transformation.

The use of dwarf varieties of agricultural plants avoids the problem of large plant size. In some cases these dwarf varieties have the added benefit of also having a rapid lifecycle. For example, the dwarf tomato, *Lycopersicon esculentum* cv. Micro-Tom (Scott and Harbaugh 1989; Meissner et al. 1997) is mutant for the SELF-PRUNING (SP) and DWARF (D) genes (Marti et al. 2006). Micro-Tom has proven extremely useful in rapidly identifying genes that control tomato development (Meissner et al. 2000; Marti et al. 2006). Thus, tomato research is no longer complicated by the problems of a large plant size and long maturity rate. More recently, Micro-Tom has been shown to be transformed efficiently (Sun et al. 2006: Orzaez et al. 2006). These improvements have made functional genomics in tomato more feasible and demonstrate how such advancements could aid *G. max* research.

Like tomato, *G. max* has the limitations of having a large size, long lifecycle and large genome. A major limitation has been its poor transformation efficiency. To address this, a variety of labs have focused their efforts on breaking down the processes that contribute to plant transformation (Parrott et al. 1989; Bailey et al. 1993; Santarem et al. 1997; Meurer et al. 2001; Tomlin et al. 2002; Ko et al. 2004; Schmidt et al. 2005). The primary process that has received a large amount of attention is efficiently obtaining somatic embryos (SEs) (Parrott et al. 1989; Bailey et al. 1993; Santarem et al. 1997; Meurer et al. 2001; Tomlin et al. 2002; Schmidt et al. 2005). Similar efforts have also been made to obtain transformed plantlets (Hinchee et al. 1988). One observation made concerning *G. max* transformation was the correlation between embryogenic potential (the ability of the *G. max* genotype to efficiently make SEs) and transformation potential (the ability of the *G. max* genotype to efficiently make SEs from transformed explants) (Ko et al. 2004). Importantly, the transformation potential of SEs, using 15 known *G. max* varieties, was shown to correlate directly to their embryogenic potential (Ko et al. 2004). However, the development of transformed SEs did not appear to correlate with the maturity group (MG) of the particular *G. max* genotype (Ko et al. 2004). Thus, the potential of a *G. max* genotype to be highly embryogeneic and/or have a high transformation potential apparently must be determined empirically for every respective genotype. This is important to truly assess its embryogenic and/or transformation potential. In those studies, Ko et al. (2004) did not compare the rate of embryogenesis between those different genotypes. Even with these advancements in somatic embryogenesis knowledge, a distinct disadvantage of *G. max* remains that obtaining plants from untransformed and transformed SEs is still a relatively slow process.

Some areas of *G. max* research do not necessarily require the recovery of whole transformed plants. For example, transferring genes by *Agrobacterium rhizogenes* results in the production of hairy roots. Thus, chimaeras having transformed roots and untransformed aerial stocks are produced. This method is rapid (~two weeks) (Collier et al. 2005) compared to methods obtaining whole transformed plants (>8 months). Accordingly, different *A. rhizogenes* strains exhibit varying levels of virulence. This is an important feature of this transformation method to take into consideration, especially if the alteration of root development or anatomy affects the process that is being studied (Sinkar et al. 1988). Hairy root transformation permits testing genes governing a variety of root processes. The added benefit is that no tissue culture is required and that aerial portions of the plant are present (Collier et al. 2005). However, hairy root transformation efficiencies differ between the different soybean genotypes (Owens and Cress 1985; Savka et al. 1990; Cho et al. 2001).

We developed a rapid cycling, dwarf *G. max* genotype through a traditional breeding program (Matthews et al. 2007) to circumvent the aforementioned problems inherent to *G. max*. Properties of *G. max* cv MiniMax are shown that potentially may make it useful as an alternative genotype for soybean research.

Materials and methods

Growth conditions

G. max cv MiniMax (PI643148) and Jack (PI540556) seeds were both grown outdoors (Beltsville, MD) and in

the growth chamber for comparative purposes. For experiments in the growth chamber, MiniMax and Jack were sown in a soil mix consisting of humus (E.C. Geiger, Harleysville, PA). The top of the rim of the pots was initially at 30 cm below the lights. Plants were grown at 14 h day length under eight standard high output cool white 48 in. fluorescent (General Electric; Fairfield, CT) and four standard 100 w incandescent lights (Phillips; New Territories, Hong Kong) in a growth chamber (model CMP 3023, Conviron Co.; Beltsville, MD). Ambient temperature was maintained at 25°C day and 20°C night. After 14 days, the photoperiod was changed to 12 h days at 25°C and 20°C night. The pots that Jack was growing in were lowered as the plants began to grow into the lights. This was done in order to prevent plant damage.

Somatic embryogenesis

Details of transformation were derived largely from the laboratory of Dr. John Finer (found at their website: http://www.oardc.ohio-state.edu/plantranslab/d20.htm) and Dr. Wayne Parrot (found at their website: http://www.cropsoil.uga.edu/soy-engineering/embryogenesisprotocol.html). Somatic embryogenesis was performed in six stages; (1) induction, (2) proliferation, (3) histodifferentiation, (4) maturation, (5) dessication and (6) germination and conversion. The duration of culture of explants into developing SEs varied, depending on the genotype studied. A range of time is provided here for induction, proliferation, histodifferentiation, maturation, dessication and germination and conversion culture. The lower values are the duration of time required for MiniMax to complete the respective phases of somatic embryogenesis while the longer times are those required for other genotypes (i.e., Williams). Pods that had emerged one to three weeks (depending on genotype) after flower formation were used as the source of immature cotyledons according to (Santarém and Finer 1999). Immature cotyledons that were 1, 2, 3, 4 and 5 mm were isolated from the pods. The immature cotyledons were then surface sterilized in 10% bleach prior to beginning somatic embryogenesis. Somatic embryo induction was done on induction medium (D40 [MSB-S, 40 mg/l 2,4-D, pH 7.0 in 0.2% gelrite]) for 2–4 weeks at 25°C (Santarém and Finer 1999). Proliferation was done on proliferation medium (D20 [MSB-S, 20 mg/l 2,4-D, pH 5.7, 5 mM asparagine in 0.2% gelrite) (Santarém and Finer 1999). The SEs were subcultured every 2–3 weeks as needed (Santarém and Finer 1999). Histodifferentiation was done on histodifferentiation medium (MSM6AC) composed of MS salts, B5 vitamins, 6% maltose, pH 5.8, 0.5% and 0.2% gelrite as a solidifying agent with 0.5% activated charcoal to remove residual 2,4-D that may interfere with the differentiation process (Ranch et al. 1986; Bailey et al. 1993). Developing SEs were cultured for seven days to four weeks as needed. Maturation was done on maturation medium (MSM6) (MS salts, B5 vitamins, 6% maltose, pH 5.8, 0.2% gelrite) for 4 days to 8 weeks. Drying was done by placing 50 mature embryos in a sealed Petri dish having a 2 × 2 × 1 cm 1% agarose cube to provide adequate humidity for 2 days to 1 week. Germination and conversion was performed on germination and conversion medium (MSO) (MS salts, B5 vitamins, 1.5% sucrose, pH 5.8, 0.2% gellan gum) for 3 days to 4 weeks. The photoperiod was left at 23 h days to prevent premature flowering. Percent conversion is the proportion of initial embryos that had matured to the cotyledonary stage and then germinated successfully (by formation of roots) and made their first trifolate leaf in soil (Ko et al. 2004). Percent conversion (out of 50 starting embryos performed in replicate) was calculated from the percent of cotyledonary stage embryos that rooted when placed on germination and conversion media after undergoing a dessication period (Ko et al. 2004). After shoot and root formation become evident, plants were transferred to Magenta boxes and subsequently into soil mix containing 75% Perlite® (E.C. Geiger) and 25% humus (E.C. Geiger) for flowering and seed set.

PCR

Plant DNA was extracted with the DNeasy Plant Miniprep Kit (Qiagen; Valencia, CA). PCR experiments were done using the following *uid*A primer pairs: forward-5′AGGAAGTGATGGAGCATCAG3′, reverse-5′CATCAGCACGTTATCGAATCC3′. Primer pairs for eGFP are forward-5′GAATTTGTTT CGTGAACTATTAGTTGCGG3′ Reverse-GCATGC CTGCAGGTCACTGGATTTTG3′. DNA for the PCR was dissociated for 10 min at 96°C, followed by PCR

cycling and temperatures set for denaturation for 30 sec at 96°C, annealing for 60 sec at 55°C and extension for 30 s at 72°C.

Agrobacterium rhizogenes transformation and maintenance

Agrobacterium rhizogenes strain K599 (K599) (Haas et al. 1995), (a generous gift from Dr. Walter Ream, University of Oregon), were transformed with the pKSF3 vector (a generous gift from I. Maiti, University of Kentucky) engineered with the figwort mosaic virus promoter sgt (FMV sgt) driving the expression of *uid*A gene encoding β-glucuronidase (GUS) (Bhattacharyya et al. 2002) in its multicloning site. This vector was further engineered to have an enhanced GFP (eGFP) (Haseloff et al. 1997) cassette driven by the *rolD* root promoter (White et al. 1985; Elmayan and Tepfer 1995) inserted into the unique *ClaI* site of the pKSF3 vector (Bhattacharyya et al. 2002). The translational terminator (t) was from CaMV35S (t-35S).

Natural resistance of K599 to various antibiotics was determined. For these experiments, untransformed K599 were grown on LB-agar containing 2, 3, 4 or 10 µg/ml tetracycline (Tet) or 30 µg/ml chloramphenicol (Chl) or 100 µg/ml spectinomycin (Spec) or 50 or 100 µg/ml Kan or 30 µg/ml rifampicin (Rif).

The freeze-thaw method (Hofgen and Willmitzer 1988) was used for K599 transformation. Transformed K599 from the positive DNA minipreps were then used for transforming MiniMax, Williams, Peking and Kent cotyledons according to Savka et al. (1990).

Agrobacterium rhizogenes-mediated axenic hairy root transformation

Hairy root transformation was performed according to Savka et al. (1990). Briefly, *G. max* cv MiniMax, Williams, Peking and Kent were grown on full strength Gamborg's B5 plus vitamins (Gamborg et al. 1968) (Research Products International; Mt. Prospect, IL) mixed with full strength Murashige and Skoog (MS) media (Murashige and Skoog 1962) (Research Products International). The MS media containing Gamborg's B5 vitamins (MSB) included 3% sucrose (Sigma; St. Louis, MO) (MSB-S media) and 0.3 % gelrite (Sigma). Ten seeds of *G. max* cv MiniMax, Williams, Peking and Kent were sown per magenta box and grown under standard fluorescent lights for 4–6 days. Transformation was performed according to Savka et al. (1990). For transformation, cotyledons were excised from the developing seedling. The cotyledons were placed onto the lid of a 60 × 15 mm Petri dish. The abaxial side of the cotyledon was deeply scored 5–6 times in both longitudinal and transverse planes to expose the vascular tissue to K599. The scored cotyledons were placed into 100 × 15 mm Petri dishes abaxial side up on top of Whatman #1 filter paper wetted with dH_2O. The cotyledons were allowed to stand in the dark for 3–4 days. The plant selectable marker of pKSF3-eGFP was Kan, found to be useful for axenic hairy root selection of *G. max* (Cho et al. 2001). The cotyledons were then transferred to full-strength MSB-S media or full-strength MS- ½ B5-S or ½ MS- ½/ B5-S containing Carb (500 µg/ml) and Kan (100 µg/ml). Hairy roots were removed 7–14 days later and placed on the same media. Roots were allowed to grow until the plate had been covered with new root material. Transformed roots were passed to fresh media by cutting a 1.5–2 cm tip with emerging lateral branches and placing it onto fresh media. This occurred three times to eliminate K599. The roots then were transferred to solid media lacking antibiotics. Alternatively, roots were transferred to the same media lacking gelrite for liquid culture.

Agrobacterium rhizogenes-mediated non- axenic hairy root transformation

Seeds of MiniMax were planted in Perlite®, germinated and grown for seven days. The plants were then carefully removed from the Perlite®. The roots were subsequently washed in deionized water. The roots were excised using a razor blade while the roots were immersed in MS media containing K599 in non-selective media as previously described. The plants were then placed in 50 ml conical vials containing K599 and vacuum infiltrated for 30 min to draw the bacteria into the scored tissue. The vacuum was then removed slowly. The cut ends of the plants were then co-cultivated in K599 for an additional 6 h to ensure

infiltration of the K599 into the wound site. After 6 h, the cut ends of the plants were placed 3–4 cm deep into fresh Perlite®. The root-less plants were covered with plastic wrap or a semi-transparent plastic lid to maintain humidity as the plants recovered. Plants were kept in the dark for two days during recovery. These methods were repeated using Fibro-gro® (Hummert International; Earth City, MO). Plants were checked for transformed roots with either a *β-glucuronidase* (GUS) stain (Jefferson et al. 1987) or eGFP for non-invasive visual screens using the same fluorescence monitoring apparatus as Alkharouf et al. (2007). Briefly, fluorescence was monitored on the GFP filter setting on a Nikon SMZ 1500 stereomicroscope (Nikon Corporation; Tokyo, Japan). Stereomicroscope images were captured with an Optronics MagnaFire model S99802 CCD camera (Optronics; Goleta, CA). GUS activity was monitored in transformed tissue by vacuum infiltration with 500 µl of GUS stain (2 mM 5-bromo-4-chloro-3-indolyl glucuronide, 100 mM potassium phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100) for 1 h. Tissue was subsequently incubated at 37°C overnight to promote development of the GUS stain reaction.

Nematode culture and plant inoculation

Plant and nematode materials were grown at the USDA Soybean Genomics and Improvement Laboratory as described previously (Klink et al. 2007a, b). Replicate experiments using different isolations of NL1-RHp and TN8 *H. glycines* J2 nematode populations (Klink et al. 2007a, b) and MiniMax seeds were repeated at different times. Briefly, MiniMax (PI643148), Williams (PI 548631), Peking (PI 548402) and Kent (PI 548586) seeds were surface sterilized, then germinated on water agar plates for three days at ambient room temperature in the dark. Hatching was promoted by incubating eggs from *H. glycines* populations NL1-RHp or TN8 in sterile water at ambient temperature on a rotary shaker at 25 rpm. After 2 days on the rotary shaker, the J2s were collected. The J2s were then concentrated by centrifugation to approximately 3,000 J2/ml. Roots were inoculated with the J2s. Nematode infection was allowed to proceed for approximately 1 month. At various stages of infection, roots were excised and prepared for acid fuchsin staining (Byrd 1983). Nematode population studies were performed using the methods of Rao Arelli (1994).

Results

Growth chamber culture conditions

MiniMax was generated through a traditional breeding program specifically for molecular and genetic applications (Matthews et al. 2007). A comparison was made to determine how MiniMax grew under growth chamber, greenhouse, and field conditions (i.e., at Beltsville, MD). Growth rate in field plots outside of Beltsville, MD were beyond the scope for this study and were not performed because MiniMax was intended solely to be a research plant for indoor growth. As compared to cv. Jack, (Fig. 1a) MiniMax is smaller in size (Fig 1b).

Somatic embryogenesis

The somatic embryogenic potential in MiniMax was evaluated. Putative SEs (those initially appearing to be *bona fide* SEs) had to satisfy three criteria to be considered SEs. Firstly, MiniMax SEs were compared to and had to appear like other published

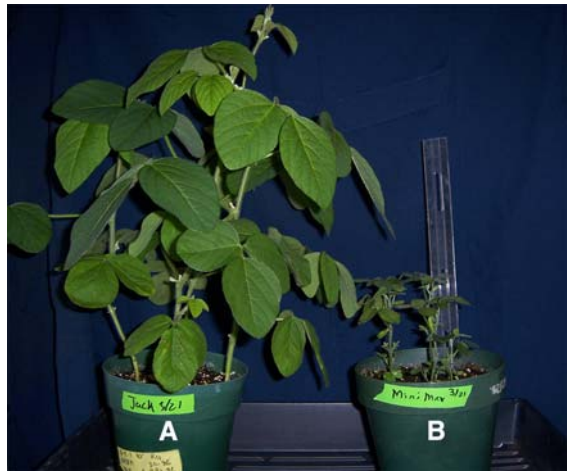

Fig. 1 Comparison of *G. max*, cv MiniMax and Jack grown in a growth chamber. (**a**) Jack is just beginning to flower; (**b**) MiniMax with maturing pods (length of ruler in [**b**] is 30 cm)

reports of SE development in other *G. max* varieties (see Materials section on somatic embryogenesis). Secondly, the progression of MiniMax SE development successfully through to the subsequent stage of tissue culture demonstrated that the minimum physiological requirement was met. The third criterion was that the SEs ultimately developed into plants. SE clusters of both MiniMax and Williams were developed and maintained according to the methods of Santarém and Finer (1999). Williams was selected for comparative purposes because SEs develop readily from their immature cotyledons. The effect of cotyledon size on the propensity of immature cotyledons to make SEs was tested. Immature MiniMax cotyledons of different sizes (2, 3, 4 and 5 mm) were placed on embryogenic media. Immature cotyledons either less than 1 mm or over 5 mm did not develop SEs in our studies. Therefore, they were not evaluated further. A comparative analysis of the ability of SEs to develop from immature cotyledons of MiniMax and Williams (Fig. 2) was made. SEs that developed from immature cotyledons were green as compared to the surrounding yellow cotyledonary tissue at the 14 day time point (Fig. 3). Comparatively fewer SEs were observed for MiniMax (Fig. 3a) than Williams (Fig. 3b) at 14 days. However, MiniMax SEs appeared more advanced in terms of their development. Those more advanced MiniMax SEs had a cup-shaped morphology that was apparent distally (Fig. 3a, tissue within the circle). The cup-shaped morphology was not apparent in Williams (Fig. 3b). By 30 days, MiniMax SEs were larger and appeared more advanced in terms of their development (Fig. 3c, tissue within the circle) as compared to Williams (Fig. 3d). MiniMax SEs from the 14 day time point consistently appeared more similar to Williams SEs at the 30 day time point. SEs were excised and grown from both the 14 and 30 day time point for both MiniMax through the proliferation (Fig. 4a), histodifferentiation (Fig. 4b), maturation (Fig. 4c), dessication (Fig. 4d) and finally the germination and conversion stages (Fig. 4e). Those plantlets were then transferred to soil mix and grow until they developed seeds (Fig. 4f) Those seeds were germinated, making offspring that then grew in a manner similar to normal plants.

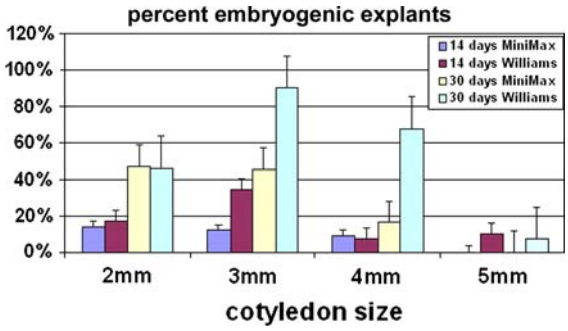

Fig. 2 Formation of SEs as a function of immature cotyledon size. The same immature cotyledon was followed from the 14 day to the 30 day time point as shown in Fig. 3

Dessication is a step required for roots to develop from *G. max* SEs. A determination of how long of a dessication period was required for ample root development was made. This showed that a large proportion of MiniMax SEs would subsequently develop roots during the germination and conversion stages after a 3 day dessication period (Fig. 5). Roots began to emerge from the plantlets in just as little as two days when transferred from the dessication stage to the germination and conversion medium (data not presented).

Hairy root transformation-axenic conditions

Some experiments were complicated by observations that *A. rhizogenes* strain K599 (K599) was resistant to antibiotics that are commonly used as bacterial selectable markers. Thus, the nature of K599 antibiotic resistance was explored (Table 1). This permitted the development of vectors suitable for K599 transformation.

MiniMax seeds were grown axenically in MSB media supplemented with 3% sucrose identically to those of Savka et al. (1990). Williams (MG-3), Peking (MG-4) and Kent (MG-4) were selected for comparative purposes because they represented the range of successful hairy root transformation (Williams>Peking>Kent). Cotyledons of MiniMax, Williams, Peking and Kent were excised and transformed using K599 harboring pKSF3 with the FMV sgt promoter (Fig. 6a) driving the expression of the *uid*A reporter gene (Bhattacharyya et al. 2002) (Fig. 6b). In some experiments, an eGFP reporter cassette was used for non-invasive visual screening of transformed tissues (Fig. 6c). Roots that exhibited the characteristic hairy root morphology developed from the cotyledons

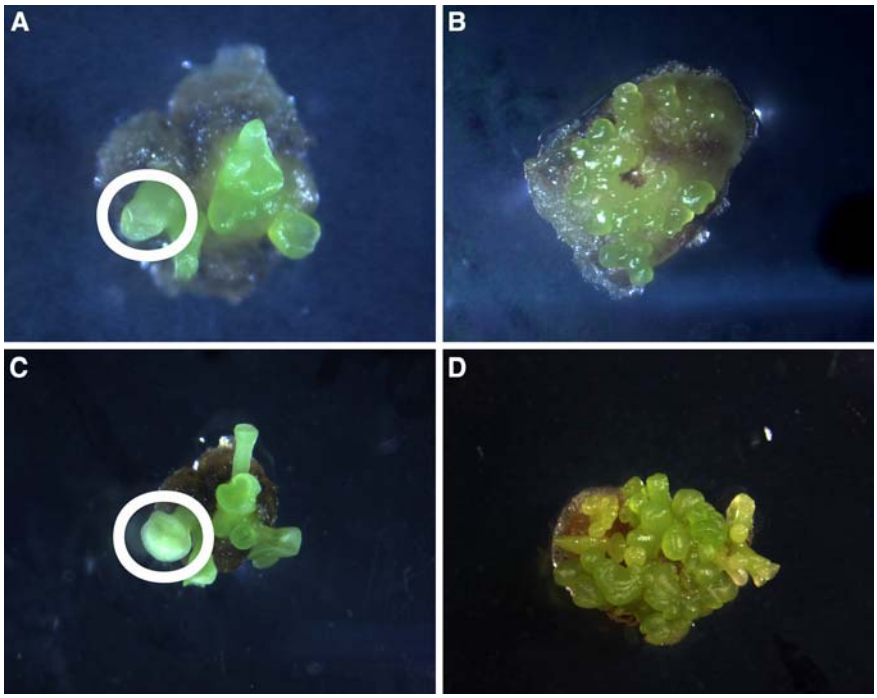

Fig. 3 Comparison of somatic embryogenesis between two *G. max* genotypes. Figures (**a**), (**b**), (**c**) and (**d**) show SEs on D40 induction medium. Figures **a** and **c** are the same MiniMax immature cotyledon photographed at the same orientation at 14 and 30 days, respectively. Figures (**b**) and (**d**) are the same Williams immature cotyledon (although Fig. (**d**) is taken at a slightly different orientation) photographed at 14 and 30 days, respectively. (**a**) MiniMax SE at 14 days, white circle = cup-shaped SE; (**b**) Williams SE at 14 days; (**c**) MiniMax SE at 30 days, white circle = cup-shaped SE; (**d**) Williams SE at 30 days

of MiniMax, Williams and Peking after 7 days of culture. Kent took much longer (2–3 weeks) to produce roots after a period of substantial callus development (data not shown). After the formation of hairy roots, the root sections (~2–2.5 cm), having lateral roots, were excised and transferred to new media. After 14 days the hairy roots of MiniMax enlarged circumferentially and subsequently developed into callus (data not shown). This was unlike hairy roots of Williams, Peking and Kent. Reducing the MS and B5 by one-half, while maintaining the sucrose conditions promptly resulted in normal MiniMax hairy root cultures (data not shown).

Hairy root transformation-non-axenic conditions

Tissue culture was avoided by adapting a non-axenic hairy root culture method (Collier et al. 2005). Plants grown in Perlite® were placed in MS media containing K599 transformed with pKSF3-eGFP (Fig. 6c). Roots were removed from the plants in the presence of K599 transformed with pKSF3-eGFP. Root primordia appeared by seven days after infection with K599 transformed with pKSF3-*uid*A (Fig. 7a). An average of 45.33% (±8.81%) plants (three replicates of 50 plants) had transformed roots. Root primordia transformed with pKSF3-eGFP exhibited positive eGFP fluorescence (Fig. 7b). Root primordia also exhibit positive GUS staining after 7 days (Fig. 7c) and hairy roots positively staining for GUS were apparent at 2 weeks after the initial K599 infection (Fig. 7d). Co-expression of both eGFP and GUS demonstrate that those respective regions of the expression vector are not interfering with each other. Hairy root anatomy was similar to untransformed roots (data not presented).

Discussion

MiniMax culture

These experiments focus on a new diminutive *G. max* genotype (MiniMax) that was developed over a period of several years through a breeding regimen (Matthews et al. 2007). MiniMax is small and through a somatic embryogenesis process produces SEs rapidly, allowing for its regeneration. Thus, it can be used specifically for molecular and genetic work. The transformability and diminutive nature of MiniMax make it possible to test the function of large numbers of genes in a relatively small amount of space compared to other *G. max* varieties. These

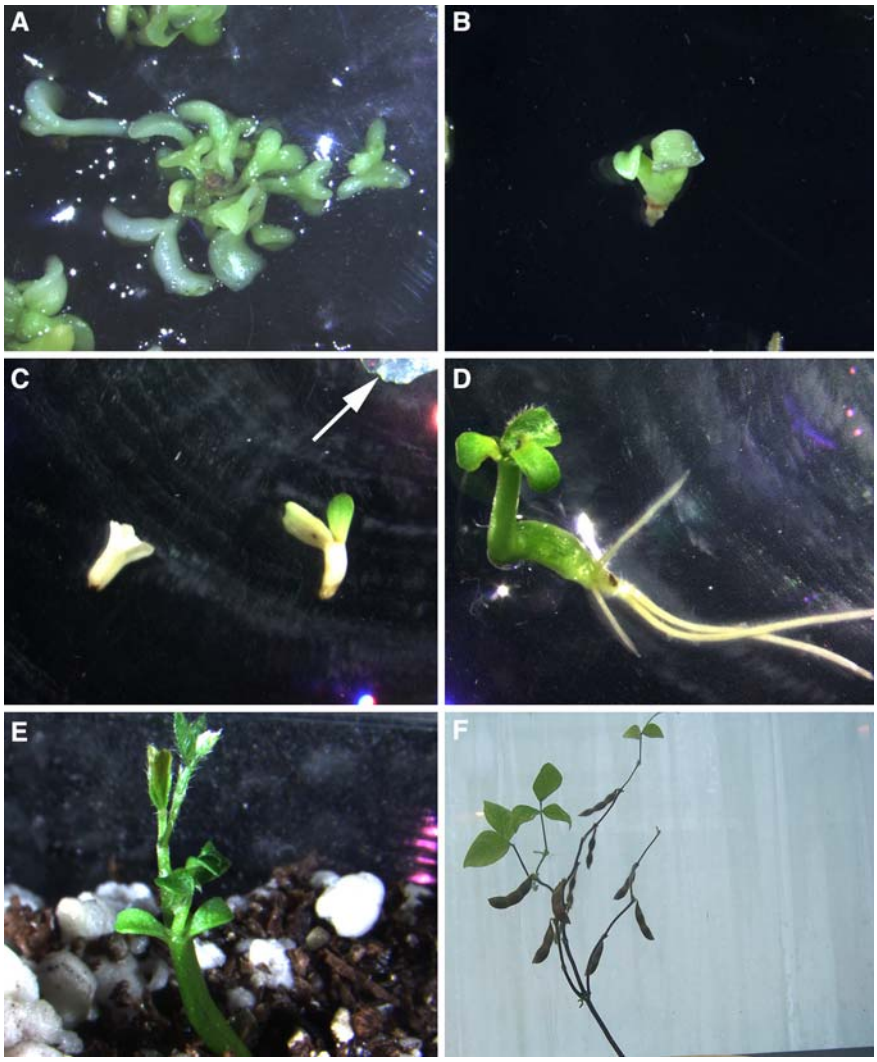

Fig. 4 Progression of MiniMax somatic embryogenesis to maturity. (**a**), SE on proliferation media; (**b**) SE on histodifferentiation media; (**c**) SE dessicating in a Petri dish with a 2 × 2 × 1 cm agar cube (arrow) to maintain humidity; (**d**) SE forming roots after five days of culture on germination and conversion medium; (**e**) plantlet with trifoliate leaf after 10 days of culture on 75% Perlite® and 25% soil in a Magenta box; (**f**) mature plant grown from a SE

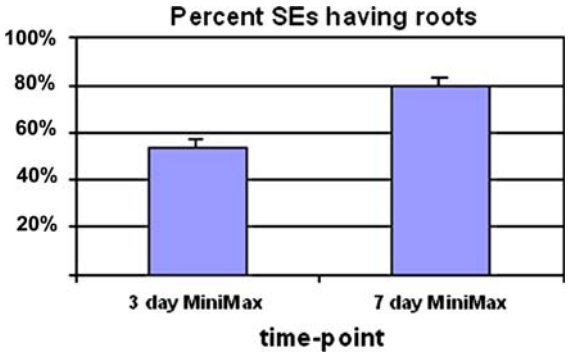

Fig. 5 Percent SEs having roots after dessication for three and seven days. MiniMax SEs were dessicated and transferred to germination and conversion medium and allowed to root. The numbers are the percent out of 50 SEs for each replicate

attributes may make MiniMax much like other dwarf varieties of agricultural plants that have shown promise for their use in molecular research (Meissner et al. 1997, 2000; Frantz et al. 2004; Frantz and Bugbee 2004; Sun et al. 2006; Orzaez et al. 2006).

Growing plants in an environmental chamber allows for the homogenization of climate conditions for year-round culturing. With a life cycle to physiological maturity of ~49–60 days in the growth chamber, the growth of ~6 generations of MiniMax, annually, are possible. It is possible that light quality/duration and or temperature play important roles in the growth habit of MiniMax. Growth habit and seed set has been shown to be influenced by light quality and duration in other dwarf varieties of agricultural

Table 1 natural resistance of *A. rhizogenes* K599 to antibiotics

Antibiotic	Concentration (μg/ml)	Sensitivity to antibiotic
Tetracycline	2	R
Tetracycline	3	S
Tetracycline	4	S
Tetracycline	10	S
Chloramphenicol	30	R
Spectinomycin	100	R
Kanamycin	50	S/R
Kanamycin	100	S
Rifampicin	30	S/R

The concentration of the antibiotic is in μg/ml. The sensitivity to antibiotic is defined in terms of resistance or susceptibility of *A. rhizogenes* to the antibiotic. Resistance and susceptible are the presence and absence of *A. rhizogenes* colonies, respectively. R = Resistant, S = Susceptible

plants such as *Oryza sativa* "Super Dwarf" (Frantz et al. 2004). However, comparing year round culture of MiniMax in the greenhouse with summertime field experiments demonstrate that they grow at similar rates. Plant density could also affect growth habit. For example, plant density had little effect on sowing to anthesis and sowing to fruit ripening for the dwarf tomato genotype Micro-Tom. However, it had a detrimental effect on plant yield, number of fruit and seeds per plant. Growth chamber conditions were maintained stringently during the course of our experiments. Using these conditions in the greenhouse may not yield similar results due to the potential for localized fluctuations in humidity, daytime fluctuations in light quality and temperature or more importantly, exposure to undesirable ambient light at night. MiniMax, however, maintains its diminutive stature and rapid cycling both in the greenhouse and in field experiments (Matthews et al. 2007).

Somatic embryogenesis of MiniMax

Ample development of SEs from immature cotyledons was observed. Although it appears that the total number of SEs that develop for MiniMax immature cotyledons are lower than that observed for Williams, those SEs of MiniMax develop more rapidly than Williams through the induction stage of tissue culture. Thus, the rapid development observed for whole MiniMax plants also appears to carry over into its tissue culture. The rapid development of SEs was observed for every step of the tissue culture process. For example, MiniMax produces SEs with a cup-shaped appearance after only 14 days of tissue culture. These SEs were ready for proliferation at this time and subsequently exhibited robust proliferation. Also, instead of requiring seven days of drying before germination and conversion can occur, a high percentage of SEs develop roots after only a three day dessication period. This period can be decreased to as

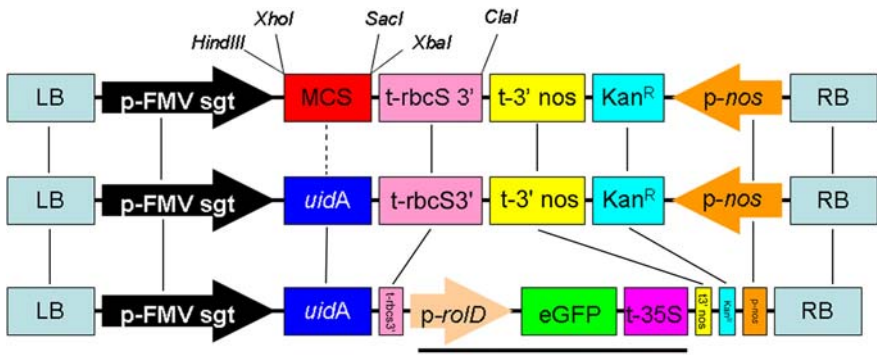

Fig. 6 Vectors used in this study. In (**a**)–(**c**) the vector components are color coded: light blue: left and right borders (LB, RB, respectively); black: FMV sgt promoter (p-FMV sgt); dark blue: GUS gene (*uid*A); red: multicloning site (MCS); pink: rbcS 3′ terminator (t-rbcS 3′); yellow: 3′ nos terminator (t-3′ nos); aqua: nptI kanamycin resistance gene (Kan^R); green: enhanced green fluorescent protein gene (eGFP); light orange: *rolD* promoter (p-*rolD*); magenta: 35S terminator (t-35S); orange: nos promoter (p-*nos*). The solid black lines between the vectors demarcate the relative positions of the vector components. The dashed black line indicates that insertion of the *uidA* gene at the MCS. (**a**) pKSF3 (Bhattacharyya et al. 2002); (**b**) pKSF3 containing the *uidA* gene in the multicloning site; (**c**) pKSF3 containing eGFP with the *rolD* promoter (underlined) driving its expression

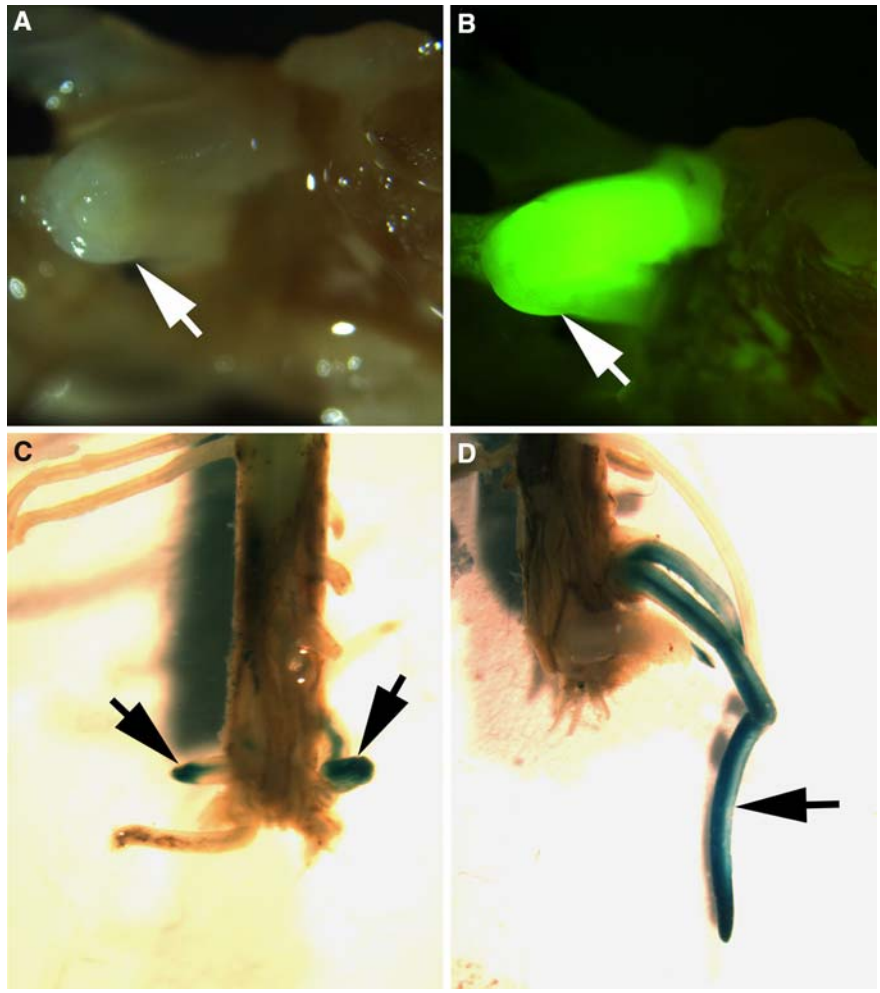

Fig. 7 Formation of chimaeras in non-sterile conditions. (**a**) light micrograph of a root primordium (arrow) one week after initial K599 infection. (**b**) The same root primordium as in (**a**) that is expressing eGFP (arrow) driven by the *rolD* promoter. (**c**) Transformed root primordium exhibiting positive *uid*A gene expression one week after initial K599 infection. (**d**) Transformed root exhibiting positive *uid*A gene expression two weeks after initial K599 infection

little as 1.5 days (data not shown). However, there is a decrease in the ability of the SEs to root. Thus, SEs develop rapidly from immature cotyledons and the percent conversion (Ko et al. 2004) to plantlets is high. These properties of MiniMax could prove as an additional advantage of the system for *G. max* research because whole plants of adequate size and maturity can be obtained rapidly for experiments in whole plants.

Hairy root transformation-axenic conditions

Many *G. max* varieties exhibit various hairy root transformation efficiencies. Owens and Cress (1985) reported that not all *G. max* varieties are equally capable of hairy root transformation. These experiments were demonstrated further by Savka et al. (1990) and repeated by Cho et al. 2001 using 10 *G. max* varieties, respectively. This variability in hairy root production between different *G. max* genotypes made testing in MiniMax relevant. MiniMax is capable of hairy root transformation. Initially it appeared that MiniMax may not be suitable for hairy root transformation because the seeds (and hence cotyledons) were small and potentially difficult to handle. However, as the seeds germinated, the small cotyledons experienced extensive expansion, resulting in cotyledons large enough for hairy root transformation according to the method of Savka et al. (1990). The use of K599 harboring the plant transformation vector pKSF3 with the FMV sgt promoter driving expression of the *uid*A reporter gene provided

strong root expression in *G. max* as previously reported for tobacco (Bhattacharyya et al. 2002). The use of an eGFP expression cassette driven by the *rolD* promoter to monitor transformation permits the easy identification of transformed roots. The added benefit is that overexpression (Cheon et al. 1993) and RNAi (Limpens et al. 2004) constructs can be added to the eGFP-containing vector backbone allowing for a variety of molecular studies to be accomplished in rapid timeframes. The ability to obtain whole plants from *A. rhizogenes* transformed roots (Tepfer 1984; Zdravkovic-Korac et al. 2004; Crane et al. 2006) could be an additional method to obtain whole transformed MiniMax plants in relatively rapid timeframes.

A problem encountered during the axenic hairy root transformation process was the development of root tissue into callus. The dwarf nature and maturity group of MiniMax may individually or both be contributing to this developmental characteristic. Other genotypes chosen for the study that are not dwarf or rapid cycling never produced callus. Importantly, the callus could re-differentiate and form new roots if callus formation was not too extensive. Decreasing the concentration of both the MS and B5 components of the culture media allowed hairy roots that had been developing into callus to produce lateral roots that were normal in root morphology and anatomy. The nature of callus formation was not explored further since it was beyond the scope of this analysis. However, callus formation may be important for efforts in regenerating whole plants from hairy roots (Tepfer 1984; Zdravkovic-Korac et al. 2004; Crane et al. 2006)

Hairy root transformation-non-axenic conditions

The production of chimaeras in non-axenic conditions was explored. These conditions were similar to those of Collier et al. (2005) with some modification for MiniMax. The use of a different growth media was found to aid in the transformation method. An advantage was using Perlite® rather than Fibrogro® because Perlite® could be removed easily from the roots for visual inspection while Fibrogro® was cumbersome to remove from the rootstock and often resulted in undesired root breakage that occurred during its removal from the plant stock. Perlite® also allowed for the removal of all non-transformed advantageous roots more readily than the method using Fibrogro® as the growth substrate.

MiniMax and its use in studying root pathogens

MiniMax is capable of compatible reactions with several *H. glycines* races including PA2, PA3, VL1, TN8 and NL1-RHp. However, for molecular analyses of *H. glycines* development, it would be important to reliably transform MiniMax. Complications concerning the strength and reliability of expression of constructs driven by the 35S CaMV promoter during *H. glycines* infection have been observed (Bertioli et al. 1999). The production of transgenic axenic hairy root cultures of MiniMax using the pKSF3-*uid*A expressing vector (Bhattacharyya et al. 2002) allowed for obtaining reliable transgene expression. *H. glycines* was shown to complete its lifecycle in about a month in hairy root cultures. This is compatible with the timeframe for *H. glycines* to complete its lifecycle in untransformed MiniMax roots. Therefore, the hairy roots did not detrimentally affect the ability of *H. glycines* to perceive the hairy root or alter the timeframe that its lifecycle is completed.

Conclusion

Technical applications of a new *G. max* genotype (i.e., MiniMax) are presented. Many of the features of MiniMax may make it a technical advancement that could be useful for both researchers interested in studying the molecular, cellular and developmental biology as well as the genetics of *G. max*. Unlike studies that use heterologous systems to study *G. max* genes, information generated from the use of MiniMax should more closely relate to field grown varieties of soybean.

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